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Response of a Generalist Herbivore *Trichoplusia ni* to Jasmonate-Mediated Induced Defense in Tomato

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Abstract The up-regulation of plant defense-related toxins or metabolic enzyme binding proteins often leads to a negative effect on herbivorous insects. These negative effects can manifest themselves at three points: changes in food ingestion, post-ingestive-changes, and post-digestive changes. Many studies have related the decrease in herbivore growth and/or survival with expression of chemicals that interfere with post-digestive effects such as the anti-nutritive effects of protease inhibitors. Nevertheless, it is unclear whether such compounds impact herbivores via earlier ingestive processes. We addressed this question by using a jasmonate-deficient mutant (Def-1), a jasmonate-overexpressor mutant (Prosystemin or Prosys), and wild-type tomato in three trials with 5th instar *Trichoplusia ni*. Decreases in relative growth rate (RGR) confirmed that *T. ni* fed on the Prosys plants developed poorly compared to those feeding on Def-1 plants (larvae on wild-types were intermediate). Preingestive and postingestive processes contributed to this effect. Total food ingested and the consumptive index were 25% lower on Prosys plants compared to Def-1 plants. Post-ingestive processes, measured by approximate digestibility, were 62% greater on Prosy plants**.** Post-digestive efficiency measured by conversion of ingested and digested food (ECI and ECD) decreased on Prosys plants two-fold compared to Def-1 plants. This post-digestive interference correlated well with the 2-fold decrease in activity of digestive enzymes, serine proteases, in Prosys-fed *T. ni* compared to those on Def-1 plants. No difference in detoxifying enzyme activity was detected.

Key Words Jasmonic acid · Induced plant response · Tomato · *Trichoplusia ni* · Prosystemin · Def-1 mutant · Nutritional index · Serine protease · Detoxification enzyme

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Introduction

The induced responses in plants to herbivore and pathogen attack are well documented and often have strong negative consequences for herbivore survivorship, growth and reproduction (Green and Ryan 1972; Karban and Myers 1989; Farmer and Ryan 1992; Stout and Duffey 1996). The negative effects of plant responses can be based on lower nutritional value or more direct toxicity (Duffey and Stout 1996; Barbehenn et al. 2007). In the latter case, a toxic effect would be caused by direct interference with midgut processes or normal life processes (Duffey and Stout 1996). In the former case, many herbivores compensate for sub-optimal nutritional value by increasing food consumption or switching to an alternative food (pre-ingestive effects), altering utilization of the food (post-ingestive effects) or increasing the efficiency of conversion of digested food to solid body matter (post-digestive effects) (Hägele and Rowell-Rahier 1999). Reduced efficiency can be divided further into either: 1) an anti-digestive effect caused by a reduction in the enzymatic conversion of ingested food; or 2) an anti-nutritive effect that reduces the physical availability or chemical composition of the food. An example of an anti-digestive effect is when protease inhibitors (PIs) from the plant reduce the protease activity

in the insect gut, thus affecting the metabolism rate of proteins and the availability of amino acids (Chen et al. 2005). An example of an anti-nutritive effect occurs when polyphenol oxidases (PPOs) cause irreversible covalent modification of amino acids and plant proteins by quinines or quinine-generated reactive oxygen species (Mahanil et al. 2008).

The defense pathway regulated by jasmonic acid (JA) is known to be one of the plant's most important lines of defense against insect herbivores (Farmer and Ryan 1992; Lightner et al. 1993; McGurl et al. 1994; Howe et al. 1996). This pathway influences many different properties in Solanaceous plants, including the formation of defense proteins (PIs), oxidative enzymes (PPO), glandular trichome density, small compounds such as alkaloids (glycoalkaloids in potato and nicotine in tobacco), and volatiles (mono- and sesquiterpenes in tomato). These compounds are known to be inducible (Ryan 2000; Thaler et al. 2002; Boughton et al. 2005; Halitschke and Baldwin 2005; Wasternack 2007). Tomato leaves contain α-tomatine, chlorogenic acid, and rutin shown to be toxic to *Heliothis zea* at foliage concentrations (Elliger et al. 1981), but it has not been determined whether these are induced through the JA pathway. Because of this, plants with an activated jasmonate pathway may vary both in nutritive content as well as levels of toxins. Nutritional quality of the plant may be altered by either changes in the amount of nutrients present or in the presence of compounds that reduce the digestibility of nutrients by the insect. While it has been shown that herbivores feeding on plants with an induced jasmonate response often have severely reduced performance, it has not been investigated to what extent these negative effects on herbivores are due to decreased nutritional availability or increased toxicity.

Modeling the function of JA has been facilitated through the use of tomato mutants or genetic manipulation of wild-type plants to alter the JA biosynthetic pathway. Jasmonate-deficient (Def-1) tomato mutants have an altered octadecanoid pathway converting linolenic acid to JA resulting in a 3-fold lower induction of JA 90 min after wounding compared to wild-type (WT) plants (Howe et al. 1996). As a result, there is a 10-to >25-fold lower accumulation of wound-inducible proteinase inhibitors (PI) in the Def-1 wounded leaves compared to WT plants. The expression of a PI-II related gene, cathepsin D inhibitor (CDI), in Def-1 plants is also less than 10% of those in WT plants (Li et al. 2002). Mortality of *Spodoptera exigua* that fed on Def-1 plants was 30% less, and larval mass of survivors was double that of larvae that fed on WT tomatoes (Thaler et al. 2002). In a second transgenic tomato line (Prosys), prosystemin is over-expressed through the use of a promoter leading to the constitutive overproduction of JA, leading to an overproduction of the defense proteins, PPO (Howe and Ryan 1999), and PIs (McGurl et al. 1994), including arginase and threonine deaminase (Chen et al. 2005). In unwounded Prosys leaves, the typical levels of PI-I, PI-II, and PPO are 14.7x, 19.1x, and 69x greater than in the unwounded WT leaves, respectively (Howe and Ryan 1999). As a result, when thrips and spider mites fed on prosystemin-overexpressing tomatoes, there is reduced damage to the plant and decreased herbivore fecundity compared to WTs (Li et al. 2002). Compared to a wild-type tomato, a Prosys mutant that overexpresses arginase had dramatically higher leaf arginase activity, reduced *Manduca sexta* larval weight gain and leaf consumption, and the level of Arg in the *M. sexta* midgut was reduced significantly (Chen et al. 2005). Jasmonate-induced levels of PIs and PPO work in combination to either modify the dietary protein or bind and inhibit digestive enzymes in the caterpillar midgut, respectively (Chen et al. 2005).

In the present study, the cabbage looper *Trichoplusia ni* (Hbn.) (Noctuidae), an insect with a large range of host plants, was used to investigate the digestive and physiological response to variable JA expression in tomato. The tomato mutants Def-1 and Prosys provide excellent models for unraveling whether the JA-induced defenses act pre-ingestive, post-ingestive, or post-digestive. The objectives of this study were to examine the difference in digestive and physiological responses of *T. ni* that are indicative of: 1) consumption and digestibility of plant nutrients (e.g., leaf area consumed, mass gain, frass production, and nutritional indices); and 2) insensitivity to plant proteins via gut serine proteases, and detoxification by gut monooxygenases, esterases, and transferases. The expectations were that tomato plants expressing higher levels of JA will: 1) have increased levels of anti-digestive and anti-nutritive compounds that will lead to significant differences in the insect's post-digestive nutritional indices; and 2) have increased concentrations of toxic allelochemicals that will lead to significantly greater detoxifying enzymes in the insect.

Methods and Materials

Plant and Insect Material Jasmonic acid deficient (Def-1), wild-type (var. Castlemart) and prosystemin over-expressing 35S::Prosystemin (Prosys) mutant tomatoes were obtained from Gregg Howe, Michigan State University. Def-1 plants were backcrossed 5 times to the Castlemart variety and were homozygous for a mutation within the jasmonate pathway leading to lower JA production (Howe et al. 1996) and a reduced ability to induce proteinase inhibitor I and II (Lightner et al. 1993). The Prosys transgenic tomatoes were developed through an agrobacterium-mediated transformation of var. Better Boy (McGurl et al. 1994), and, as a

consequence, accumulate high levels of wound response proteins in the absence of wounding (Howe and Ryan 1999). Seeds for the Prosys plants were collected from a homozygous line backcrossed to var. Castlemart (Li et al. 2002). All tomato plants were grown in 4″ diam pots with Metromix potting soil in the greenhouse from seed. Plants were used when they were between 4 and 5 wk in age.

Cabbage looper *Trichoplusia ni* larvae were obtained from Benzon Research (Carlisle, PA, USA). Larvae were reared on multispecies diet (Southland Products, Lake Village AR, USA) until the 5th instar. All larvae used in trials were mid 5th instar. Larvae were reared at 26-28°C, 16:8 hr L:D.

Tomato—T. ni Greenhouse Trials Three greenhouse tomato trials were conducted between October 2006 and January 2007. The plants were grown in the greenhouse at 27°C under natural light conditions with supplemental lighting until 22:00 hr. Fertilizer (21:5:20 N:P:K, 150 ppm N) was added weekly to the pots. Fifty Def-1, WT, and Prosys plants without prior herbivore damage were selected for use in the trials. The third leaf from the top was selected for the insect feeding trial. Outlines of the first five leaflets from the leaf were obtained from each plant by using an underlying sheet of white paper and an overlying acetate sheet. A clear plastic clip cage (approx. 5 cm diam.) then was attached to the first or terminal leaflet of the leaf. When all leaflets were traced, a 6–8 hr-starved, pre-weighed *T. ni* larva was placed in each clip cage. After 5–6 hr, the clip cage was moved to an adjacent leaflet on the same leaf, and this was repeated up to three more times over a 24 hr period. The cage was moved depending on the degree of damage per leaflet. If the leaflet was largely or completely consumed by the larva, the cage was moved. If <50% of the leaf area was removed, the cage was not moved. At the end of the trial, the remaining leaf tissue was scanned (Canoscan, Canon, Burlington NJ, USA) and compared to a scan of the leaflet tracing in order to calculate the actual leaf area consumed. Each time a cage was moved the frass produced by the insect was collected.

After 24 hr, all larvae were removed from the cages and held in cups on ice. In the laboratory, the final fresh mass of each larva was measured. Ten of the 50 larvae were randomly selected for measurement of dry mass, the remainder were divided into three groups for enzyme assays. To determine dry mass, larvae were removed from the ice, starved at room temperature for 1–2 hr, weighed, and then frozen at −20°C. Later these larvae were dried at 65°C for 3 d and reweighed. Larvae used in the enzyme assays were placed on ice, and the midgut was removed by dissection as described below. Calculation of the dry mass of the tomato leaflet area consumed was based on dry mass of discs cut from 10–15 leaflets per variety with a #10 cork borer and dried for 3 d at 65°C. These leaf discs were cut from 10 plants, and the disc was cut from the base or the widest part of the leaflet. The surface area damaged on each leaflet was converted to dry mass based on the mean mass per disc for each tomato variety. Initial dry mass of the insects was calculated by multiplying the initial fresh mass by the ratio of the mean final dry mass : fresh mass. Frass collected from individual insects was dried at 65°C for 3 d and weighed.

Nutritional Analysis The nutritional indices calculated for the *T. ni*—fed tomato mutants and wild-type were as follows: relative growth rate (RGR), consumption index (CI), approximate digestibility (AD), efficiency of conversion of digested food (ECD), and efficiency of ingested food (ECI). The indices were calculated according to the Waldbauer method as described in Slansky and Scriber (1985) and Rayapuram and Baldwin (2006). CI [(leaf ingested)/(larval mass gain x number of days)] provides a measure of the total amount of leaf consumed relative to the body mass gained during the 1 day feeding period. Increases in CI have been associated with compensation by nutritionally limited insects. AD [(leaf mass ingested—frass mass)/(leaf mass ingested)] is the difference between the amount of food consumed and the amount of frass produced per unit food consumed. This provides a measure of the efficiency of digestion of the ingested food. ECD [(larval mass gain)/(leaf mass ingested—frass mass)] and ECI [(larval mass gain)/(leaf mass ingested)] provide a measure of the efficiency of conversion of absorbed food and ingested food, respectively. The latter 2 indices measure the mass increment of the insect relative to the amount of food absorbed and eaten, respectively. Lower ECI and ECD indicate that insects are not getting essential macronutrients, whereas a change in AD reflects different mechanisms occurring after ingestion, but prior to digestion. RGR (growth attained per unit of body mass per unit of time) is the product of the relative consumption rate (RCR=mg consumed per mg gained per day) and nutritional indices (RGR=RCR x ECI) (Schoonhoven et al. 2005).

Total Serine Protease Activity Individual *T. ni* midguts were dissected on ice, and the whole midgut was placed in a preweighed cryovial with 100 µl of MilliQ water and reweighed. Samples then were frozen at−80°C until the protease assay was performed. The frozen whole midguts were thawed on ice, transferred to a 1.5 ml centrifuge tube, and additional distilled water was added, based on the midgut mass in order to have a 10% midgut solution. The midgut then was processed using a disposable pellet mixer (VWR) with pestle to fit the 1.5 ml microtube. The 10% homogenate was used to measure total serine protease

activity for 10 individual *T. ni* larvae per tomato variety per trial. Total midgut serine protease activity was quantified with azocasein (Sigma) substrate using a method adapted from Hegedus et al. (2003) (P. Wang, personal communication). The midgut homogenate (20 μl) was added to 150 μl of 1% azocasein solution in pH 11 glycine-sodium hydroxide (0.05 M). All samples were incubated at 28°C for 15 min. TCA (10%) was added (170 μl) and incubated for 1 h at room temperature. The sample was centrifuged at 13,000 RPM for 10 min, and the supernatant was collected. Sodium hydroxide (1 M) was added (340 μl), and the absorbance was read at 495 nm with a Beckman spectrophotometer (Li et al. 2004). Total midgut protease activity is calculated by subtracting the azocasein blank absorbance from the azocasein sample absorbance divided by the incubation time in min multiplied by 1000 ([(Abs $_{(sample)}$—Abs $_{(blank)}$]/min * 1000). The units are tryptic activity (mU) per min of incubation per mg insect body weight (mU/min/mg bw). Leaf homogenate from each of the tomato types also was assessed for protease activity using the above method to ensure that no background protease activity was present due to plant material present in the midguts.

Previous studies have shown that adaptation by insects to protease inhibitors (PIs) in their diet involves increases in the amount of PI-insensitive proteases (Broadway 1995; Jongsma and Bolter 1997). One method of examining the difference in the profile of proteases in the midgut is to apply a protease inhibitor with specific activity. Bowman-Birk soybean protease inhibitor (BBI) (Sigma) was selected as it targets trypsin and chymotrypsin proteases, the principal serine proteases in lepidopterans (Abdeen et al. 2005; Chougule et al. 2008). In order to test the hypothesis that *T. ni* larvae adjust to increasing levels of JA-induced defenses by up-regulating insensitive proteases, differences in the remaining amount of serine protease activity was measured between BBI-incubated midgut homogenates taken from *T. ni* that fed on the three tomato mutants in trial 1 relative to the homogenates incubated without BBI. The midgut homogenates of the treated *T. ni* were incubated for 15 min with BBI over a range of concentrations (0.1, 0.5, 1, 2, and 8 mg/ml) that showed a linear inhibition of protease activity with azocasein substrate with increasing inhibitor concentration until saturation was approached. Total serine protease activity was measured as described above, and the percent inhibition caused by the BBI incubation was determined.

Cytochrome P450/b_5 Assay Microsomes were prepared from the dissected midguts of 20 *T. ni* larvae fed on the wild-type and two tomato mutants. The midguts were dissected on ice and rinsed in Rinaldini's saline. The 20 midguts were combined and homogenized in 20 ml 0.1 M sodium phosphate homogenizing buffer (pH 7.5) (Lee and Scott 1989). Homogenates were centrifuged at 10,000 RPM for 20 min, the supernatant was transferred to a 30 ml centrifuge tube and centrifuged at 100,000 RPM for 1 h. The microsome pellet was homogenized in a resuspension buffer (pH 7.5) (Lee and Scott 1989) and frozen at−80°C until the assay was performed. Cytochrome P450 and b_5 were determined following the methods described for house fly (Lee and Scott 1989). Microsome protein was determined by the Bradford (1976) method.

Glutathione S-transferase Assay Individual *T. ni* midguts were dissected on ice, rinsed in Rinaldini's saline, and homogenized in 500 μl of sodium phosphate buffer (pH 7.5). Ten individuals per treatment from each of the three trials were analyzed. A disposable pellet mixer was used to grind the midgut-buffer mixture. After homogenizing the midgut, a further 500 μl of buffer were added so that the final preparation was one midgut per 1 ml buffer. Samples were centrifuged at 10,000 RPM, and the supernatant was transferred to 1.5 ml vials. Samples were kept frozen at−80°C until both the glutathione *S*-transferase (GST) and esterase assays were conducted. The substrates, 150 mM 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma) (1 μl), and 150 mM 1,2-dichloro-4-nitrobenzene (DCNB) (Sigma) (1 μl) were used with 15 mM reduced glutathione prepared in pH 7.0 potassium phosphate buffer (50 μl) and homogenate (25 μl). The methods were adapted from Baker et al. (1998) where three replicates were prepared for each midgut sample on a 96 well plate on ice. Readings at 340 nm were taken every 30 sec for 10 min at 25°C using a microplate reader (Thermo Fisher Scientific, Waltham MA, USA). The initial and final readings used for GST activity were at 3 and 8 min (5 min interval) after the initiation of the plate readings. Blank wells were prepared by replacing the homogenate with pH 7.0 buffer. Ten individuals per treatment from each of the three trials were analysed. Protein concentration was determined by the Bradford (1976) method.

Esterase Assay Midgut supernatants were used for the determination of esterase activity. The esterase substrates used were α-and β-naphthyl acetate (NA) and 4-methylumbelliferyl acetate (4-MUA), and a microplate assay was adapted from the methods described by Baker et al. (1998). For the α-and β-NA assays, supernatant was diluted 1:100 with pH 7.0 buffer. Into each plate well we added: 110 μl of buffer, 50 μl of either 4 mM α-or β-NA dissolved in acetone, and 40 μl of diluted sample. After 15 min at 25°C, a stop reagent, 25 μl of 0.8% Diazo Blue in 3.4% SDS was added, followed by a further 10 min wait before measuring the activity at 595 nm (α-NA) or 490 nm

(β-NA). Each plate was set up with triplicate wells of samples, standards with either α-naphthol or β-naphthol (4–40 μM) in buffer and blank wells (buffer only). Esterase activity with all three substrates was standardized to protein concentration.

Statistical Analyses ANCOVA was used to analyze each of the nutritional indices to avoid problems with the statistical analysis of ratios (Raubenheimer and Simpson 1992) (Proc GLM SAS ver 9.1). In all cases, the independent variables are plant type and trial. For RGR, final mass is the dependent variable and initial mass is the covariate. For CI, plant mass consumed is the dependent variable, and initial mass is the covariate. For AD, the plant mass consumed minus the frass mass produced is the dependent variable, and the mass consumed is the covariate. For ECD, the larval mass gain is the dependent variable, and amount consumed minus the frass produced is the covariate. For ECI, the larval mass gain is the dependent variable, and the amount consumed is the covariate. In the case of AD, ECI, and ECD, the interaction between the plant-type and the covariate was tested. Two-way ANOVA was used to analyze the biomarkers (protease, cytochrome P450, cytochrome b_5, esterase, and GST activity) for the three separate trials. Results were combined and analyzed, and the difference between the treatments was compared among and between the trials. Comparison of treatment means used Tukeys (SAS ver.9.1, SAS Institute, Cary NC, USA). Data were log + 1 transformed, and the normality and homogeneity of all data sets were confirmed (SAS ver.9.1). The effects of the sbBBI treatment on protease activity were ARC Sine (Squareroot) transformed and analyzed using one-way ANOVA (SAS ver. 9.1).

Results

Insect Relative Growth Rate, Leaf Area Consumption, Frass Production, and Nutritional Indices Plant-type had a large effect on caterpillar performance with a significantly lower relative growth rate (RGR) when comparing larvae on Prosys plants to those on Def-1 and WT plants (Fig. 1a). *Trichoplusia ni* caterpillars ate 27–31% less on Prosys plants compared to the Def-1 and WT plants (Table 1). The consumptive index (CI) decreased by 25% when larvae fed on Prosys plants and were compared to those on Def-1 plants; larvae feeding on WT plants showed a CI similar to those feeding on Prosys plants (Fig. 1a).

Post-ingestive efficiency, measured as approximate digestibility (AD), was 62% less on Def-1 plants and 34% less on wild type plants compared to Prosys plants. The caterpillars on Def-1 plants ate more, but also produced more frass (Table 1).

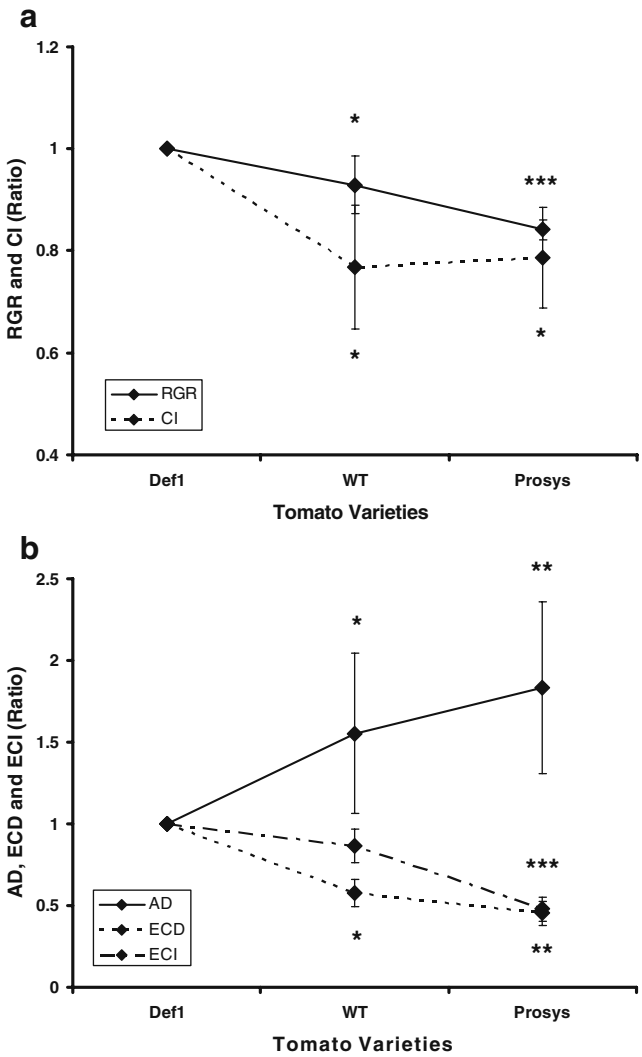

Fig. 1 Mean ratio (± S.E.) for Def-1:Def-1, WT:Def-1 and Prosys:Def-1 of the following: **a** relative growth rate (RGR) and consumption index (CI); and **b** approximate digestibility (AD), efficiency of conversion of digested food (ECD), and efficiency of conversion of ingested food (ECI) for *Trichoplusia ni* feeding for 24 hr on three tomato mutants. RGR, CI, AD, ECD, and ECI ratios marked with either a *, ** or *** indicates that there was a significant difference (*LS Means*, *ANCOVA*, $P<0.05$) between the Def-1 and WT or Def-1 and Prosys mutants in 1, 2, or all of the 3 trials, respectively. $N=27$ (Def-1); 28 (WT) and 28 (Prosys)

In contrast, post-digestive measures of efficiency of food conversion (ECD and ECI) were 2-fold higher for caterpillars fed on Def-1 plants compared to those feeding on WT or Prosys plants (Fig. 1b). The nutritional indices of caterpillars feeding on WT plants were typically intermediate to those of larvae feeding on Prosys and Def-1 plants. (Table 2).

Total Serine Protease Activity Total serine proteases were highest in *T. ni* feeding on the Def-1 plants, intermediate on WT, and lowest on the Prosys plants (One way ANOVA:

Table 1 Mean total dry mass of tomato leaf consumed and dried frass produced by *Trichoplusia ni* feeding for 24 hr on Def-1, wild-type (WT) and Prosys tomato mutants

Plant	Leaf mass[a] mg (S.E.)	Ratio[b] (S.E.)	Frass mass mg (S.E.)	Ratio (S.E.)
Def-1[c]	15.23 (1.34)^{A}	1	10.34 (0.49)^{A}	1
WT[d]	9.7 (0.65)^{A}	0.69 (0.16)	7.14 (0.46)^{A}	0.74 (0.14)
Prosys[e]	10.36 (0.78)^{A}	0.73 (0.14)	6.17 (0.49)^{B}	0.62 (0.13)

[a] Mean (± S.E.) leaf and frass dry mass values having same upper case letter are not significantly different (*two way ANOVA*: $F_{8,69}$=5.57 and $F_{8,69}$=13.62, respectively; *Tukey's* P>0.05).

[b] Mean ratio (± S.E.) for Def-1:Def-1, WT:Def-1 and Prosys:Def-1 are provided for leaf and frass mass.

[c] N=27.

[d] N=28.

[e] N=28.

$F_{8,72}$=13.73; Tukey's P<0.05, Fig. 2). The serine protease activity in leaf samples of all tomato types was 5% or less of the midgut protease activity for larvae feeding on either plant type (data not shown), and therefore cannot be responsible for the observed results.

The estimated value of IC_{50} for Bowman-Birk Inhibitor (BBI) on the midgut total serine protease activity was 2 mg/ml for the insects feeding on WT plants. Saturation of the BBI inhibitor occurred at 8 mg/ml and before 50% inhibition of the Prosys-treated protease midgut activity was reached. At the WT-fed *T. ni* BBI IC_{50} concentration, the level of inhibition was 44% and 31% for the Def-1 and Prosys *T. ni*, respectively (Fig. 3). At this concentration there was greater inhibition of the total serine protease activity in the WT vs. the Prosys *T. ni* midguts (One way ANOVA: $F_{14,30}$=12.86; Tukey's P<0.05). Since BBI is a protease-specific inhibitor, this result indicates that *T. ni* larvae might modify BBI-insensitive proteases to adapt to increasing PI levels in the Prosys plants.

Detoxification Enzyme Activity The total P450 and cytochrome b_5 levels in *T. ni* larvae were unaffected by feeding on the different mutant tomato plants (One way ANOVA: $F_{8,15}$=7.45 and $F_{8,\ 14}$=4.06, respectively; Tukey's P>0.05). No significant differences in the CDNB GST or DCNB GST activity were observed for *T. ni* feeding on the three tomato mutants (One way ANOVA: $F_{8,171}$=61.95 and $F_{8,183}$=5.88, respectively; Tukey's P>0.05).

In general, the *T. ni* 4-methylumbelliferyl acetate esterase, α-and β-naphthyl acetate activity were not affected by plant type (One way ANOVA: $F_{8,\ 180}$= 274.06, $F_{8,195}$=124.49 and $F_{8,243}$=69.21, respectively; Tukey's P>0.05). However, activities of all three larval esterases were significantly higher (Tukey's P<0.05) on the Prosys plants compared to esterase activities of larvae on the other two tomato plants in one of the trials (a different trial for each esterase).

Discussion

Although effects of plant defenses were detected at all stages of caterpillar feeding, they appeared most strongly at the post-digestive stage. Overall caterpillar performance was greater on the Def-1 plants as seen in the significantly higher relative growth rate compared to larvae on WT and Prosys plants. This was due in part to an approximately 30% greater consumption on the Def-1 plants. The consumption index (CI) was lower on Prosys compared to the Def-1 plants, but no different from the wild type plants.

A post-ingestive measure of efficiency, approximate digestability (AD), was greater for *T. ni* that fed on Prosys compared to those on Def-1 plants. This may be a result of the reduced fecal output, likely due to a lowering of the food conversion rate in the midgut of the caterpillars feeding on Prosys plants. The increase in AD may compensate partially for the 2-to 3-fold decrease in efficiency of conversion of both digested (ECD) and ingested food (ECI). These results are comparable with a 3 d trial where *T. ni* larvae fed on Def-1, Prosys, and wild-type tomato plants (unpublished results, J. Thaler). The RGR and ECD for Prosys-fed *T. ni* were much less than the Def-1-fed *T. ni* after 3 d compared to 1 d of feeding, but the relative ECI between the two mutants was the same for the two feeding periods.

Insect larvae often compensate for detrimental digestive effects of their food plants by increasing the approximate digestibility (AD). However, over an 11 d trial, *M. sexta* larvae showed greater leaf consumption and ECD on the LOX3 silenced transgenic *Nicotiana attenuata* (Solanaceae) plants than on WT plants, but AD was no different (Rayapuram and Baldwin 2006). These results are similar in that both *M. sexta* and *T. ni* increase leaf consumption and increase digestibility on jasmonate-deficient plants. However, our study found lowered AD values in larvae feeding on Def-1 and WT plants compared to Prosys plants,

Table 2 Analysis of covariance for *Trichoplusia ni* feeding for 24 hr on Def-1, wild-type (WT) and Prosys tomato mutants

	df[a]	*F*	*P*
Relative Growth Rate (RGR)			
Initial dry weight (DW)	1	287.7	<0.001
Plant type	2	27.4	<0.001
Trial	2	39.2	<0.001
Trial x Plant type	4	5.3	0.001
Error	9,73	53.6	<0.001
Consumption Index (CI)			
Initial DW	1	12.1	0.001
Plant type	2	15.1	<0.001
Trial	2	9.3	<0.001
Trial x Plant type	4	6.4	<0.001
Error	9,73	9.5	<0.001
Approximate Digestability (AD)			
DW leaf consumed	1	231.7	<0.001
Plant type	2	14.1	<0.001
Trial	2	35.6	<0.001
Trial x Plant type	4	5.2	0.001
DW leaf consumed x Plant type[b]	2	7.9	0.001
Error	11,71	63.2	<0.001
Efficiency of Conversion of Digested Food (ECD)			
DW leaf consumed—frass produced	1	1.3	0.268
Plant type	2	10.1	<0.001
Trial	2	30.7	<0.001
Trial x Plant type	4	4.5	0.003
(DW leaf consumed—frass) x Plant type	2	1.9	0.161
Error	11,71	13.2	<0.001
Efficiency of Conversion of Ingested Food (ECI)			
DW leaf consumed	1	22.3	<0.001
Plant type	2	12.8	<0.001
Trial	2	40.5	<0.001
Trial x Plant type	4	2.6	0.039
DW leaf consumed x Plant type	2	8.2	0.001
Error	11,71	19.3	<0.001

[a] The degrees of freedom, *F* and *P* values are adjusted for the covariate.

[b] The covariate by Plant type interaction for AD and ECI was significant ($P<0.05$), but not for ECD ($P>0.05$)

whereas the *M. sexta* study did not detect any difference in AD in dependence of the food plant. This difference between *T. ni* and *M. sexta* responses to plant types could be due to different jasmonate-regulated traits in tobacco compared to tomato or to differences in the generalist feeder *T. ni* and the specialist feeder, *M. sexta*. In addition, trial length influences the effect of plant type on larval performance and digestion parameters. We observed also that lengthening the feeding period from 1 to 3 d for *T. ni* on tomato WT, Def-1 and Prosys mutants resulted in

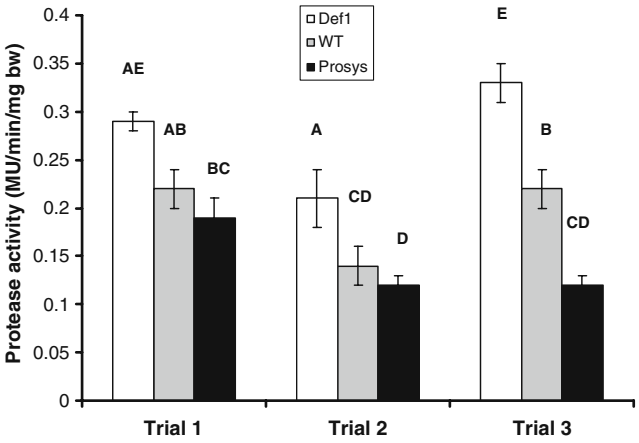

Fig. 2 Serine protease tryptic activity (mU) per min of incubation per mg insect body weight (mU/min/mg bw) in 5th instar *Trichoplusia ni* larvae 24 hr after feeding on three tomato JA mutants in three separate trials. Bars with different shading and the same upper case letter indicate no significant difference between mutants in each trial (two-*way ANOVA*, $P>0.05$). Bars with the same shading and the same upper case letter indicate no significant difference in protease activity for the same mutant between trials (two-*way ANOVA*, $P>0.05$). $N=28$ (Trial 1); 25 (Trial 2), and 28 (Trial 3)

significantly reduced growth and ECD on the Prosys plants, but no difference in AD was detected (unpublished results, J. Thaler).

Further support for the importance of post-digestive stage interference is the reduced serine protease activity in Prosys-fed *T. ni*, compared to those fed on Def-1 plants. There is an inverse relationship between the *T. ni* midgut serine protease levels after 24 hr feeding and the reported levels of defense proteins and enzymes in induced Def-1, WT, and Prosys plants (Howe et al. 1996; Howe and Ryan

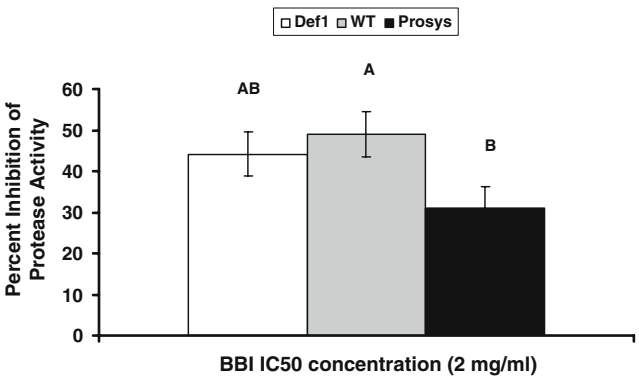

Fig. 3 Percent inhibition of serine protease activity in 5th instar *Trichoplusia ni* larvae after midgut homogenates were incubated with Soybean Bowman-Birk Inhibitor (BBI). Bars with different shading and the same upper case letter indicate no significant difference between mutants (*one way ANOVA*, $P>0.05$). $N=3$ midgut samples per plant type

1999; Li et al. 2002). An inverse correlation between the level of foliar PI activity and larval growth has been observed similarly with both *T. ni* fed on cabbage and *S. exigua* fed on tomato (Broadway et al. 1986; Broadway and Colvin 1992). Prosys plants have constitutively greater levels of PIs, PPO, and other compounds well-characterized by many researchers, so the reduction of serine protease levels in the *T. ni* midguts is just one measurable response that confirms a direct effect of plant nutrients on the insect metabolism. Insects other than the cabbage looper, such as the tomato hornworm, may be more successful herbivores on plants that express higher JA-inducible defenses through their greater ability to compensate for reduced protease levels.

A small compensation for post-digestive interference was detected through the use of the soybean Bowman-Birk inhibitor (BBI) in the present study. To our knowledge this is the first study to examine the effect of BBI on *T. ni* serine protease activity post-feeding on plants expressing a range of PI activity. According to Chougule et al. (2008), BBI has dual specificity towards tryptic and chymotryptic activities. Although the relative amount of trypsin-chymotrypsin activity inhibited by BBI was less in the midguts of the *T. ni* feeding on the Prosys tomatoes, the serine protease activity was reduced to a greater degree than in the midgut of *T. ni* fed on the other two tomato plants. Since we did not isolate the trypsin-like enzymes from *T. ni* that fed on the three tomato mutants, we cannot confirm an increase in the relative proportion of resistant trypsin-like enzymes in the midgut of *T. ni* that fed on the Prosys plants. However, the lower proportion of trypsin-chymotrypsin activity reduced by BBI in the Prosys-fed *T. ni* does agree with the results of a study by Broadway (1995) where *T. ni* fed a diet with cabbage proteinase inhibitors had increased levels of activity from enzymes that were less susceptible to inhibition.

Up-regulation of other proteases may require more than 24 hr, and thus may not be detectable in the WT-fed *T. ni* samples. An increase in the level of proteases with a lower affinity for BBI or the presence of other classes of proteolytic enzymes was thought to be the reason for the lower BBI-inhibitory activity in *Heliothis obsolete* feeding on hemizygote transgenic tomato plants compared to control plants (Abdeen et al. 2005). Those authors suggested that the 1.6-fold increase in BBI-insensitive digestive proteases in *H. obsolete* was a compensation for the partial loss of activity caused by the presence of the introduced serine protease inhibitors in the transgenic tomatoes. The differences between the two studies suggests that BBI may have an effect on a different range of trypsin proteases present in *T. ni* compared to *H. obsolete*, some of which might be up-regulated in response to induced tomato PI levels. Another possibility may be that the protein substrate casein does not reflect quantitatively the activity of the PI-insensitive proteases (Jongsma and Bolter 1997).

Among the many JA-pathway inducible compounds, PPO has a direct effect on digestive processes in insects by decreasing the availability of essential amino acids in the insect gut, considered to be an anti-nutritive effect (Barbehenn et al. 2007). This is different from the effect produced by PIs, since a reduction in protease activity is considered an anti-digestive effect. Despite these differences, tomato mutants that overexpress PPO had a similar negative effect on insect growth, consumption rate, ECD, and ECI (Mahanil et al. 2008) observed in the present study.

A toxic effect or combined mechanism of action that negatively impacts the post-digestive processes cannot be ruled out. However, there was no difference in the levels of cytochrome P450/b_5 and GST, and only small, inconsistent increases in esterase activity in the *T. ni* that fed on the Prosys tomatoes. Induction of insect monooxygenases by phenolic compounds such as indoles and flavones has been described by Yu (1984). However, for both a specialist and a generalist insect fed on transgenic tobacco lines, no correlation between foliar levels of phenolics (chlorgenic acid, rutin, and total flavonoids) and larval growth and survival was noted (Bi et al. 1997). Chlorogenic acid, rutin, and α-tomatine are phenolics found at toxic levels in tomato leaves (Elliger et al. 1981), but detoxification enzyme levels have, to our knowledge, never been measured after insect exposure to these compounds. In selected plant systems, induced monooxygenase or glutathione levels can play a role in the insect response to plant toxins, for example induced GST activity as a generalized detoxification response to plant allelochemicals (Yu 1982, 1984; Wadleigh and Yu 1987; Francis et al. 2005). In the present study, there was no affect on the levels of cytochrome P450/b_5 or glutathione transferases associated with feeding on the three tomato mutants. This confirms the importance of proteins as an induced defense in tomato, rather than alkaloids, in order to reduce herbivory.

We conclude that pre and post-ingestive processes work together to reduce insect growth on plants with high levels of jasmonate regulated defenses. JA-mediated plant responses directly impact midgut protein digestion at least in part through increased defense proteins (PIs) and oxidative enzymes (PPO). In this study, the reduction in serine proteases and the ability of the insect to absorb nutrients for growth was decreased significantly in defended plants, as exemplified by the lower efficiency of conversion of absorbed and ingested food on Prosys-fed versus Def-1-fed *T. ni*. Based upon what we know of the post-digestive stage, a reduction in ECI and ECD indicates that the insect is not able to obtain the necessary essential

macronutrients for growth. In the present case, we have empirical evidence that suggests an anti-digestive effect is occurring since there is a reduction in the protease activity in the insect gut. However, evidence from prior studies with the Prosys mutant details the significantly greater production of PPO relative to the wild-type, a factor that has an anti-nutritive effect. One of the two expectations of this study was met: post-digestive processes were significantly affected by JA-induced defenses due to both anti-digestive and anti-nutritive activity, whereas the toxic affect from tomato allelochemicals did not produce a measured response in the insect.

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